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Karen L. Elkins, David A. Leiby, Robert K. Winegar
Carol A. Nacy, and Anne H. Fortier

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Walter Reed Army Institute of Research
Washington, D.C. 20307-5100

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Mice inoculated either subcutaneoulsy (s.c.) or intradermally (i.d.) with a sublethal dose of *Francisella tularensis* strain LVS are immune to a lethal intraperitoneal (i.p.) or intravenous (i.v.) challenge of LVS. Here, we show that this immunity developed quite rapidly: mice given a sublethal dose of live LVS s.c. or i.d. (but not i.v.) withstood lethal i.p., i.v., or i.d. challenge as early as two days after the initial inoculation, in spite of the presence of bacterial burdens already in tissues. The magnitude of this early protection was quite impressive. The i.p. LD_{50} in naive C3H/HeN mice was only 2 bacteria, while the i.p. LD_{50} in mcie given 10^4 LVS i.d. three days previously was 3 x 10^6 bacteria. Similarly, the i.v. LD_{50} in C3H/HeN mice shifted from 3 x 10^2 in naive mice to 5 x 10^6 in primed mice within three days after i.d. LVS infectin. Comparable changes in the i.p. and i.v. LD_{50} were observed in C57Bl/6J mice. This rapid generation of protective immunity was specific for LVS, in that mice given a sublethal i.d. inoculation of LVS did not survive a lethal challenge with either *Salmonella typhimurium* strain W118 or *Escherichia coli* 0118 strain BORT at any time, nor could mice given sublethal doses of *S. typhimurium*, *E. coli*, or *Mycobacterium bovis* strain BCG survive lethal doses of LVS. Although an

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increase in the mean time to death from *S. typhimurium* infection was noted when mice were given a sublethal i.d. dose of LVS four to fourteen days earlier, no overall increase in protection or change in the *S. typhimurium* LD_{50} was observed. Thus, sublethal infection LVS at skin sites induced rapid and specific protective immunity.

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Rapid Generation of Specific Protective Immunity to *Francisella tularensis*

KAREN L. ELKINS,* DAVID A. LEIBY, ROBERT K. WINEGAR, CAROL A. NACY, AND ANNE H. FORTIER

Department of Cellular Immunology, Walter Reed Army Institute of Research, 9620 Medical Center Drive, Suite 200, Rockville, Maryland 20850

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Mice inoculated either subcutaneously (s.c.) or intradermally (i.d.) with a sublethal dose of *Francisella tularensis* LVS are immune to a lethal intraperitoneal (i.p.) or intravenous (i.v.) challenge of LVS. Here, we show that this immunity developed quite rapidly: mice given a sublethal dose of live LVS s.c. or i.d. (but not i.v.) withstood lethal i.p., i.v., or i.d. challenge as early as 2 days after the initial inoculation, despite the presence of bacterial burdens already in tissues. The magnitude of this early protection was quite impressive. The i.p. 50% lethal dose (LD_{50}) in naive C3H/HeN mice was only 2 bacteria, while the i.p. LD_{50} in mice given 10^4 LVS i.d. 3 days previously was 3×10^6 bacteria. Similarly, the i.v. LD_{50} in C3H/HeN mice shifted from 3×10^2 in naive mice to 5×10^6 in primed mice within 3 days after i.d. LVS infection. Comparable changes in the i.p. and i.v. LD_{50} were observed in C57BL/6J mice. This rapid generation of protective immunity was specific for LVS, in that mice given a sublethal i.d. inoculation of LVS did not survive a lethal challenge with either *Salmonella typhimurium* W118 or *Escherichia coli* O118 BORT at any time, nor could mice given sublethal doses of *S. typhimurium*, *E. coli*, or *Mycobacterium bovis* BCG survive lethal doses of LVS. Although an increase in the mean time to death from *S. typhimurium* infection was noted when mice were given a sublethal i.d. dose of LVS 4 to 14 days earlier, no overall increase in protection or change in the *S. typhimurium* LD_{50} was observed. Thus, sublethal infection with LVS at skin sites induced rapid and specific protective immunity.

Francisella tularensis, the causative agent of tularemia, is a facultative intracellular bacterium found predominantly in cold weather latitudes. Infection is usually initiated through skin, blood, or aerosol contact with infected rodents, resulting in a potentially fatal ulceroglandular, respiratory, or typhoidal disease (41). An attenuated vaccine strain is available, although its success in preventing human disease in nature or in laboratory workers is variable (4, 43), and the mechanisms responsible for development of protective immunity to *F. tularensis* in humans are not well understood (41). Our previous studies demonstrated that the live vaccine strain (LVS) is pathogenic for laboratory mice when introduced intraperitoneally (i.p.), and causes a lethal infection that is quite similar to human disease (12); the mouse is thus an appropriate model for elucidating the nature of infection and immunity to *F. tularensis*. The i.p. or intravenous (i.v.) 50% lethal dose (LD_{50}) in inbred mice is quite low, generally approaching a single bacterium. The LD_{50} when LVS is introduced into mice intradermally (i.d.) at the base of the tail or subcutaneously (s.c.) in skin or in footpads, however, is several orders of magnitude higher (10, 12). The mechanisms responsible for this striking difference are the subject of ongoing study. Since initial resistance to i.d. *Francisella* infection is completely dependent on tumor necrosis factor (TNF) and gamma interferon (IFN-γ) production in the first 2 days after infection (22), the observation of a high LD_{50} after i.d. infection at least suggests that events in murine skin facilitate rapid activation of IFN-γ-producing cells that are required for both initial survival and clearance of bacteria.

Protective immunity to many pathogens is generally assessed at time points when organisms from the initial infection are already cleared, typically weeks or months after the initial infection. Previously, we studied the expression of protective immunity by challenging mice with a lethal dose of LVS 3 weeks after sublethal infection (10, 12, 22). Mice that survived a sublethal inoculum of LVS i.d. or s.c. were resistant to lethal i.p., i.v., or i.d. challenge with LVS (10, 12) by 3 weeks after sublethal priming. By this time, bacteria were usually no longer found in mouse tissues (9). When we tested the time course of development of protective immunity to LVS, we found that protection develops quite rapidly to an impressive degree: only 3 days after initiation of a sublethal i.d. infection with LVS, mice were able to survive a lethal i.p. LVS challenge of 10,000 LD_{50}s. This rapid generation of immunity is characterized in the present report.

MATERIALS AND METHODS

Mice. Specific-pathogen-free male C3H/HeNHSD mice, 5 to 6 weeks of age, were purchased from Harlan Sprague Dawley, Frederick, Md., and housed in barrier facilities until use at 7 to 10 weeks of age. Pathogen-free male C57BL/6J and BALB/cByJ mice were purchased from Jackson Laboratory, Bar Harbor, Maine. All mice were quarantined for 1 week after transportation before use and were age matched within an experiment. Sentinel mice were routinely screened serologically for evidence of infection with a panel of mouse pathogens and were consistently found to be negative.

Bacteria. *F. tularensis* LVS (ATCC 29684) was purchased from American Type Culture Collection, Rockville, Md., and cultured in either supplemented Mueller-Hinton broth in a 37°C air shaker or on modified Mueller-Hinton agar plates in a humidified 37°C incubator with 5% CO_2 (2, 12). Stock cultures of LVS were grown overnight in broth from a single

* Corresponding author.

isolated colony to the stationary phase and frozen in broth (without the addition of glycerol) in aliquots at −80°C. Viable CFU after freezing were determined by plate counts of serial dilutions, as necessary. Bacteria were heat killed by incubation of the diluted bacterial stock at 56°C for 60 min; bacterial death was confirmed by plating of an aliquot. *Escherichia coli* BORT was a gift from Alan Cross (Walter Reed Army Institute of Research), and *Salmonella typhimurium* W118 was a gift from Samuel Formal (Walter Reed Army Institute of Research). Both were propagated either in L broth or on L agar plates and similarly frozen without the addition of glycerol. *Mycobacterium bovis* BCG 179 was purchased as lyophilized cultures from the Swiss Serum Institute; dilutions were based on viable CFU reported by the manufacturer. For inoculation of mice, bacterial cultures were thawed or reconstituted with sterile water immediately before use and diluted appropriately in phosphate-buffered saline (PBS); actual numbers of bacteria inoculated were confirmed by plate counts at the time of injection.

Inoculations. Mice were given various doses of LVS i.p., i.v. via the lateral tail vein, i.d. at the base of the tail, or s.c. in either the footpad or in a skin flap on the lower right flank. Survival was monitored for 30 days, although most deaths occurred within 4 to 7 days after infection. The LD_{50} under various experimental circumstances was determined by inoculation of groups of at least five mice with doses of bacteria covering a 4-log range and calculated by the method of Reed and Muench (35).

Estimation of bacteria in organs. At various times after infection with 10^4 LVS i.d., mice were euthanized by cervical dislocation. Peritoneal fluid was recovered by injection of 5 ml of sterile PBS into the exposed peritoneal cavity and withdrawal of fluid; generally 3 to 4 ml of fluid was recovered. Spleens were removed aseptically and homogenized in 5 ml of sterile PBS. Cells from either source were lysed by the addition of sodium dodecyl sulfate (final concentration, 0.05%), and numbers of bacteria were determined by plate counts of appropriately diluted samples of each tissue. Results are expressed as the mean CFU per organ for groups of three mice; standard errors are omitted for clarity but were generally less than 20%.

RESULTS

Time course of development of protective immunity to *F. tularensis*. To characterize the time course of development of protective immunity, we inoculated mice with a sublethal dose (10^3) of LVS i.d. (priming); initial dose response studies established that priming with sublethal doses of LVS i.d. ranging from 10^2 to 10^4 resulted in the generation of solid protective immunity (see Table 4). A lethal dose (10^4) of LVS i.p. (challenge) was then given on days 1, 2, 3, 7, and 21; this challenge dose is 4 logs greater than the i.p. LD_{50} for naive (unprimed) mice (10, 12). The results of this experiment are shown in Fig. 1. On day 1 after priming, all challenged mice died. As observed previously (10, 12), all mice challenged 3 weeks (day 21) after priming remained alive for greater than 30 days. Surprisingly, 75% of mice challenged on day 2 survived lethal i.p. challenge, and 100% of mice challenged on days 3 and 7 survived (Fig. 1). Substantial protective immunity was thus expressed quite rapidly after sublethal inoculation with LVS, at a point when appreciable numbers of bacteria (on the order of 10^5 bacteria per spleen or liver; see reference 12) were still present in mouse tissues from the initial inoculation (9, 12).

The extent of bacterial dissemination in the peritoneum and in the spleen after sublethal i.d. inoculation is shown in Table 1. Bacteria were found in the spleen within 1 day after i.d. inoculation with 10^4 LVS, and by day 3 after inoculation, bacteria were observed in the peritoneum. Bacterial burdens peaked by day 4 in both sites and began to decrease thereafter. Comparable results were observed when 10^3 bacteria, the other commonly used priming dose, were inoculated i.d. (with correspondingly lower absolute numbers of bacteria detected in organs [9]). Thus, i.p. challenge with a lethal dose of 10^4 LVS on days 3 and after added bacterial numbers to a preexisting bacterial burden already present in the peritoneum and elsewhere (12) as a result of the initial i.d. infection.

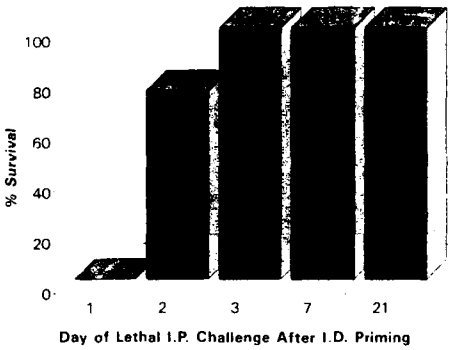

FIG. 1. Resistance to i.p. LVS infection after i.d. priming is generated rapidly. Groups of five BALB/cByJ mice were primed with 10^3 LVS i.d. on day 0 and challenged with 10^4 LVS i.p. (■) on days 1, 2, 3, 7, and 21 after i.d. infection. Actual inoculation doses were confirmed by plate count at the time of inoculation. Survival was monitored daily for 30 days thereafter, although all deaths occurred within 4 to 6 days after infection. This experiment is representative of five experiments of similar design, all of which had comparable results.

Magnitude of protection against lethal *F. tularensis* challenge administered by several routes. Mice were given 10^4 LVS i.d. and challenged with lethal i.p., i.v., or i.d. doses of LVS 3 days later. The doses chosen were 4 logs, 3 logs, and 3 logs greater than the LD_{50} for the i.p., i.v., and i.d. routes, respectively (10, 12). As seen in Table 2, all mice (five of five) succumbed to i.p., i.v., and i.d. infection when control diluent PBS was used for priming 3 days previously. The

TABLE 1. Time course of appearance of *F. tularensis* LVS in tissues after i.d. inoculation

Day after i.d. infection	CFU/site[a]	
	Peritoneum	Spleen
1	<10[b]	2.7×10^3
2	<10[b]	1.3×10^5
3	2.5×10^3	4.6×10^6
4	4.4×10^3	5.7×10^7
5	4.5×10^2	3.2×10^6

[a] C3H/HeN mice were inoculated i.d. with 10^3 LVS. On the indicated days thereafter, three mice per time point were sacrificed; peritoneal fluid and cells were obtained by lavage with 5 ml of sterile PBS, and spleens were homogenized in 5 ml of sterile PBS. Serial dilutions of aliquots of each were plated as described in Materials and Methods. Numbers of CFU were adjusted to a total for each site; results shown are the mean CFU per site for groups of three mice. This experiment is representative of two experiments of similar design, both of which had comparable results.

[b] Limit of detection of assay was 10 colonies per organ.

TABLE 2. Rapid generation of resistance to lethal infection by i.d. priming with *F. tularensis* LVS

Sublethal priming[a]	Lethal challenge	Deaths/total
PBS i.d.	10^4 i.p.	5/5
	10^5 i.v.	5/5
	10^7 i.d.	5/5
10^4 i.d.	10^4 i.p.	1/5
	10^5 i.v.	0/5
	10^7 i.d.	0/5

[a] Groups of five C3H/HeN mice were inoculated with 10^4 LVS or the control diluent, PBS, i.d. at the base of the tail. Three days later, they were challenged with lethal doses of LVS by the indicated route; actual inoculation doses were confirmed by plate count at the time of inoculation. Survival was monitored daily for 30 days thereafter, although all deaths occurred within 4 to 8 days after infection. This experiment is representative of three experiments of similar design, all of which had comparable results.

majority (80%) of mice survived lethal i.p. challenge, and all survived lethal i.v. or i.d. challenge. Thus, the rapid generation of immunity after i.d. LVS priming is systemic and can be expressed after lethal challenge by any tested route.

The magnitude of protective immunity was assessed by determining the LVS i.p. and i.v. LD_{50} 3 days after sublethal i.d. priming. While unprimed C3H/HeN or C57BL/6J mice had an i.p. LD_{50} of only 2 bacteria, i.d. LVS priming 3 days previously increased the i.p. LD_{50} to 3×10^6 bacteria (Table 3). This dramatic shift in the LD_{50} is almost the same as previously demonstrated in long-term-primed mice: the i.p. LD_{50} in C3H/HeN mice that were i.d. infected with LVS 3 weeks previously was 4×10^7 (10). Similarly, short-term (3-day) priming resulted in a substantial shift in the i.v. LD_{50}, to 5×10^6 in C3H/HeN mice and 8×10^6 in C57BL/6J mice (Table 3). By comparison, the i.v. LD_{50} in C3H/HeN mice primed 3 weeks previously with LVS is 9×10^6 (10). Thus, most of the increase in the i.p. or i.v. LD_{50} that occurred after i.d. priming took place within the first 3 days after initial i.d. infection with LVS.

Skin-related induction of immunity to *F. tularensis*. The generation of long-term immunity to LVS is not limited to i.d. inoculation of LVS at the base of the tail, since mice can be primed to resist lethal challenge 3 weeks later by introducing LVS s.c. at other skin sites, such as in the flank or in the footpad (10). This was also true for the rapid generation of immunity, as shown in Table 4. Mice were primed i.v.

TABLE 3. Shift in LD_{50} values within 3 days after i.d. priming with *F. tularensis* LVS

Mouse strain	LD_{50}[a]			
	i.p.		i.v.	
	Unprimed	Primed	Unprimed	Primed
C3H/HeN	2×10^0	3×10^6	3×10^2	5×10^6
C57BL/6J	2×10^0	NT[b]	2×10^3	8×10^6

[a] Groups of five or six mice of the indicated strains were inoculated i.d. with 10^3 LVS (primed) or the control diluent, PBS (unprimed). Three days later, they were challenged with doses of LVS i.p. ranging from 10^0 to 10^7; actual inoculation doses were confirmed by plate count at the time of inoculation. Survival was monitored daily for 30 days thereafter, although all deaths occurred within 4 to 5 days after infection. LD_{50} values were calculated by the method of Reed and Muench (35). These values are representative of at least two LD_{50} determinations for each combination of route and mouse strain.

[b] NT, not tested.

TABLE 4. Rapid generation of resistance to lethal i.p. infection by sublethal infection with *F. tularensis* LVS in skin sites

Sublethal priming[a]	Lethal challenge	Deaths/total
PBS i.d.	10^4 i.p.	5/5
10^2 i.d. (base of tail)	10^4 i.p.	1/5
10^3 i.d. (base of tail)	10^4 i.p.	0/5
10^3 s.c. (flank)	10^4 i.p.	0/5
10^3 s.c. (footpad)	10^4 i.p.	0/5
10^2 i.v.	10^4 i.p.	4/5
10^3 heat-killed i.d.	10^4 i.p.	5/5

[a] Groups of five C57BL/6J mice were inoculated with live LVS, heat-killed LVS, or the control diluent, PBS, i.d., i.v., or s.c. as indicated. Three days later, they were challenged with a lethal dose of 10^4 LVS i.p.; actual inoculation doses were confirmed by plate count at the time of inoculation. Survival was monitored daily for 30 days thereafter, although all deaths occurred within 4 to 5 days after infection. This experiment is representative of three experiments of similar design, all of which had comparable results.

with 10^2 bacteria (the highest available sublethal dose), 10^2 or 10^3 LVS i.d. at the base of the tail (to correspond with the available i.v. dose and the previously used priming dose, respectively), 10^3 s.c. in the lower right flank or s.c. in the footpad (corresponding to the i.d. dose), or i.d. with 10^3 heat-killed bacteria or with control PBS. Three days later, they were challenged with a lethal i.p. dose (10^4) of LVS. All PBS-primed mice succumbed to i.p. LVS infection, but all 10^3 i.d. or s.c. LVS-primed mice survived (Table 4). The majority (80%) of mice given 10^2 LVS i.d. survived, but the majority of mice given the same number (10^2) of bacteria i.v. or 10^3 heat-killed bacteria i.d. died. Thus, immunity to LVS developed rapidly after infection with sublethal doses of LVS at any skin-related site.

Specificity of rapid generation of protective immunity to *F. tularensis*. The rapid expression of protective immunity, demonstrable only 2 days after priming, occurred before detectable levels of specific antibody appeared in the serum of infected mice (36), and too quickly to be attributed to activation and response of conventional T cells. The possibility that this protection was not specific for LVS was tested with several different bacterial pathogens. Mice were primed with a sublethal (10^3) dose of LVS i.d. and challenged at various times after infection with lethal doses i.p. of either LVS (10^4, 10,000 LD_{50}) or *E. coli* BORT (10^7; 50 LD_{50}s). While 100% of challenged mice survived lethal LVS infection initiated on day 2 after priming and at all subsequent time points, none of the mice reproducibly survived lethal *E. coli* challenge (Fig. 2). There was no effect of LVS priming on the mean time to death of mice that died from *E. coli* infection at any time point. Mice did survive lethal *E. coli* challenge if infected with a sublethal i.d. dose of the homologous bacteria 3 weeks earlier, indicating that protective immunity to *E. coli* could be generated by sublethal infection (Fig. 2). A reciprocal experiment demonstrated that priming mice with a sublethal i.d. dose of *E. coli* that was protective for lethal i.p. *E. coli* challenge (Fig. 2) failed to protect against lethal i.p. LVS challenge (data not shown). Thus, under these conditions there was no evidence for cross protection between LVS and *E. coli* BORT.

Further, mice were infected i.d. either with a sublethal (10^3) dose of LVS or with 10^6 *M. bovis* BCG, a potent activator of macrophages (see reference 37), natural killer (NK) cells (21), and some γ/δ T cells (6, 14, 18) which establishes a chronic sublethal infection in mice. At days 1, 3, 8, and 21 after LVS or BCG i.d. infection, mice were challenged with a lethal dose of LVS (10^4) i.p. All day 3 or

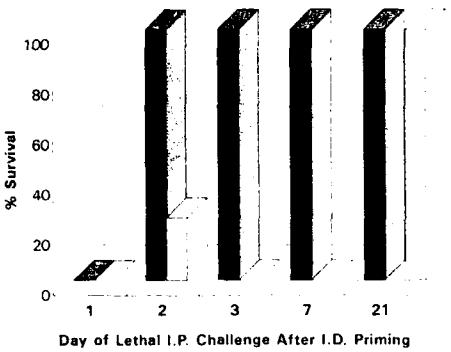

FIG. 2. Effect of i.d. LVS priming on lethal LVS and lethal *E. coli* challenge 1 to 21 days later. Groups of five C3H/HeN mice were primed with 10^3 LVS i.d. on day 0 and challenged with 10^4 LVS (■) or 10^7 *E. coli* i.p. (□) on days 1, 2, 3, 7, and 21 after i.d. infection. Other mice were given 10^6 *E. coli* i.d. on day 0 and challenged with 10^7 *E. coli* i.p. on day 21 (▨). Actual inoculation doses were confirmed by plate count at the time of inoculation. Survival was monitored daily for 30 days thereafter, although all deaths occurred within 4 to 7 days after infection. This experiment is representative of three experiments of similar design, all of which had comparable results.

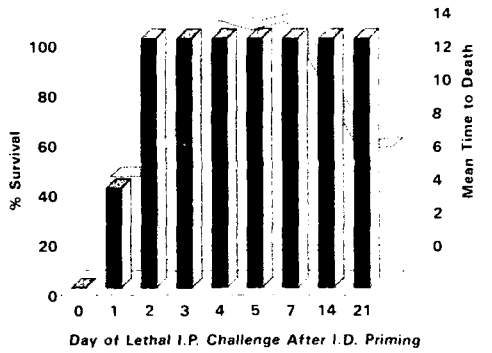

FIG. 4. Effect of i.d. LVS priming on lethal LVS or *Salmonella* challenge 1 to 21 days later. Groups of five BALB/cByJ mice were primed with 10^3 LVS i.d. on day 0 and challenged with 10^3 LVS i.p. (■) or 10^5 *S. typhimurium* i.p. (□) on days 1 to 21 after i.d. infection. Actual inoculation doses were confirmed by plate count at the time of inoculation. Survival was monitored daily for 30 days thereafter, although all deaths occurred within 4 to 7 days after infection. Mean time to death (▨) was calculated by arithmetic average of the day of death observed for individual mice within a group; mice which survived for greater than 30 days were not included in the calculation. Standard errors are omitted for clarity of presentation, but were generally less than 15%. This experiment is representative of four experiments of similar design, all of which had comparable results.

greater LVS-primed mice survived lethal challenge, while no BCG-primed mice survived (Fig. 3). Thus, protective immunity to LVS was not generated by infection with this dose of BCG, which is sufficient to prime mice for in vitro lymphokine production (29, 37). Since BCG is not virulent for mice, a reciprocal challenge experiment testing immunity to lethal BCG challenge after LVS priming was not possible.

S. typhimurium W118 is a gram-negative intracellular bacterium that, like LVS, has an i.p. LD_{50} of a single bacterium in susceptible BALB/c mice (19, 32). Mice were primed with a sublethal (10^3) dose of LVS i.d. and challenged at various times thereafter with a lethal (10^3) i.p. dose of either LVS or W118 (1,000 LD_{50}s in both cases). As seen in Fig. 4, by day 2 after i.d. infection, all mice could withstand lethal LVS challenge, but none could survive lethal W118 challenge. The only measurable influence on W118 infection was an increase in the mean time to death (Fig. 4) from about 4 days in unprimed mice to a maximum of about 14 days in mice primed 4 to 7 days previously with LVS i.d. This increase waned with time; by day 21 after sublethal i.d. LVS infection, the mean time to death from *Salmonella* infection was about 6 days. Sublethal i.d. infection with W118 did not reliably lead to protection against lethal *Salmonella* challenge, and therefore the reciprocal experiment was not interpretable.

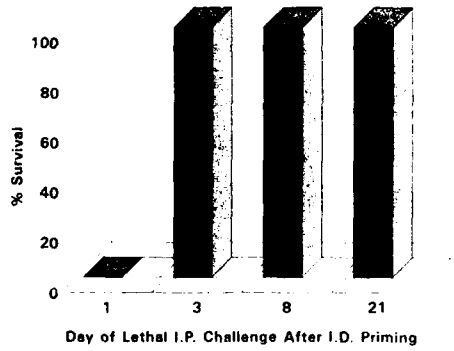

FIG. 3. Effect of i.d. LVS or BCG priming on lethal LVS challenge 1 to 21 days later. Groups of five C3H/HeN mice were primed with 10^3 LVS (■) or 10^8 BCG i.d. (□) on day 0 and challenged with 10^4 LVS i.p. on days 1, 3, 8, and 21 after i.d. infection. Actual inoculation doses were confirmed by plate count at the time of inoculation. Survival was monitored daily for 30 days thereafter, although all deaths occurred within 4 to 7 days after infection. This experiment is representative of two experiments of similar design, both of which had comparable results.

This subtle influence on the mean time to death from *Salmonella* infection after LVS priming suggested that some nonspecific resistance was generated but that it was insufficient to permit survival of a 1,000-LD_{50} lethal challenge. We therefore tested the effect on the actual W118 i.p. LD_{50} on day 4 after sublethal LVS priming i.d., a time when LVS priming resulted in the maximal increase in mean time to death from *Salmonella* infection (Fig. 4). In unprimed C3H/HeN mice, the i.p. LD_{50} for W118 was 3×10^2, while in mice primed 4 days previously with 10^3 LVS i.d., the *Salmonella* i.p. LD_{50} was 1×10^3. Conversely, there was no difference between the LVS i.p. LD_{50} values determined in unprimed mice (one bacterium) and mice primed with a sublethal dose of W118 i.d. 3 days earlier (three bacteria).

Thus, there was no effect from *E. coli* or BCG priming on lethal LVS infection nor from LVS priming on lethal *E. coli* infection and at best only a slight effect on *Salmonella* infection in terms of both mean time to death and shift in LD_{50}. Taken together, these results indicate that the rapid development of immunity after sublethal i.d. LVS priming is indeed specific for LVS.

DISCUSSION

The LD_{50} for *F. tularensis* LVS in mice is only 1 to 100 bacteria when infection is initiated by either i.p. or i.v. inoculation, but is 4 to 6 logs higher (10^4 to 10^6) when

infection is initiated by skin-related routes (i.d. or s.c. [10, 12, 22]). This disparity may have a correlate in the course of wild-type human *Francisella* infection: tularemia is estimated to be fatal in only 5% of untreated cases when the route of entry is the skin, but may approach 60% when infection is initiated by aerosol (40). Further, sublethal infection with LVS in mice leads to the development of significant protective immunity: animals given either i.d. or s.c. LVS are able to withstand lethal i.p. or i.v. challenges 4 to 7 logs higher than the LD_{50} in naive mice (10, 12). Our previous studies concentrated on the characterization of this immunity at 3 weeks after i.d. infection, a time when bacteria are usually cleared from organs. Here we document that this protective immunity develops quite rapidly and well before bacterial clearance: within 2 days after sublethal i.d. LVS infection, virtually all mice were able to survive enormous lethal i.p., i.v., or even i.d. challenges. As observed with other bacteria such as *S. typhimurium* (8, 16), i.d. infection with live bacteria was far more successful at generating immunity than inoculation with the same numbers of heat-killed bacteria (Table 4). Further, sublethal i.v. infection was unable to generate substantial immunity within 3 days, although comparable numbers of bacteria introduced i.d. were able to generate immunity (Table 4); however, it is possible that i.v. inoculation results in relatively more inactivation of injected bacteria than i.d. inoculation. Taken together, these observations suggest that live infection at skin sites is especially efficient in the rapid generation of protective immunity.

Since substantial numbers of bacteria have already disseminated to the peritoneum and the spleen within 3 days of i.d. inoculation (Table 1) (12), infection in the skin has apparently activated mechanisms that permitted survival not only of the initial bacteria burden, but also of the secondary introduction of even greater numbers of bacteria. The difference between i.p. or i.v. LD_{50}s with and without priming measured here (Table 3) is therefore an underestimation of the bacterial burdens which i.d.-primed mice can withstand.

The rapid development of protective immunity occurred before specific antibody to LVS is detected in serum (36) and before specific T-cell expansion is usually detected (25, 31). The 2- to 3-day interval necessary between priming and survival of challenge corresponds exactly, however, to the time course of tumor necrosis factor (TNF) and IFN-γ production after i.d. LVS infection (22). Thus, using inoculations of anti-TNF and anti-IFN-γ monoclonal antibody to deplete cytokines in vivo, we demonstrated previously that TNF and IFN-γ must be available within the first 2 days after i.d. inoculation of LVS for mice to survive and clear bacteria (22). It seemed likely, therefore, that the early activation of cytokine production might also be responsible for the observed rapid development of protection. The effects of such cytokine production might further be nonspecific and lead to protection against other bacteria in which TNF and/or IFN-γ play a protective role.

This prediction was not substantiated by the results presented here, however. In *E. coli* BORT infection, TNF is produced early after infection and has a beneficial role in protection against disease (5). Sublethal *E. coli* infection had no effect on survival after LVS infection, however (Fig. 2). Further, BCG infection s.c. in footpads and in the skin induces antigen-responsive α/β (14) and γ/δ (18) T cells in the draining lymph nodes of infected mice. Mycobacteria-responsive T cells produce interleukin 2 (18) in vivo and IFN-γ at least in vitro (6, 11, 34). Spleens removed from mice primed with the dose and lot of BCG used here readily secrete many cytokines, including IFN-γ, upon in vitro stimulation (29, 37). Despite probable T-cell activation and cytokine production in vivo, however, inoculation of BCG i.d. had no effect on survival of LVS infection.

Both *Listeria* (3, 7, 15, 30, 46) and *Salmonella* (17, 19, 25–28, 30, 44, 47) infections have an early, T-cell-independent phase that limits bacterial replication and a later, T-cell-dependent phase that results in actual bacterial clearance. Both IFN-γ and TNF appear to play an important role in early resistance to *Salmonella* (25, 26, 28, 38, 44, 45) and *Listeria* (3, 7, 46) infection, and the IFN-γ in both cases may be derived from a non-T NK cell (3, 7, 38, 46). Thus, nonspecific cross-protection between *Listeria* and *Salmonella* cells is predictable, and indeed about 50% of the mice infected with the *aro* avirulent SL3235 *Salmonella* strain can survive 10 to 100 LD_{50}s of *Listeria* infection (19). Nonspecific protection against *L. monocytogenes* was noted by day 3 after *Salmonella* infection that appeared to peak by day 6 and wane within 1 month (19, 47). Similar to the results for LVS reported here, substantial specific protection against homologous *Salmonella* challenge was also demonstrable within 3 days after *Salmonella* priming; this early protective activity was attributed to macrophage-like adherent cells (but not T cells) that could be detected by passive transfer experiments (19).

In the present study, the possibility of nonspecific cross-protection between LVS and *S. typhimurium* was studied carefully, since infections with *S. typhimurium* and *F. tularensis* are quite similar in many respects. Both are gram-negative facultative intracellular organisms that have a very low LD_{50} for mice when introduced i.p. and a higher LD_{50} when introduced i.d. (9, 10, 12). IFN-γ and TNF probably play important roles in early resistance to infection with both (22, 25, 26, 28, 44). *Salmonella* infection in mice is, however, under control of at least four genes in the mouse (*ity*, *lps*, *xid*, and another unidentified gene in C3HeB/FeJ mice [33]), while obvious genetic control is not a feature of *Francisella* infection. In the present study, sublethal infection with LVS had no effect on survival after challenge with 1,000 LD_{50}s of *S. typhimurium* W118 in *Salmonella*-susceptible (*ity*^{s}) mice (Fig. 4), and in fact had only a very small effect when the i.p. *Salmonella* LD_{50} was measured 3 days after sublethal LVS infection in resistant (*ity*^{r}) mice (see Results) or in susceptible BALB/cByJ mice (9). Conversely, *Salmonella* infection had no effect on the magnitude of the i.p. LVS LD_{50} (see Results). The only evidence for nonspecific cross-protection that we observed was a transient increase in mean time to death from *Salmonella* infection in mice infected 3 to 14 days previously with a sublethal dose of LVS. These increases in mean time to death were also noted in the LD_{50} studies, but no long-term effect on survival was observed. Taken together, all these results indicate that the rapid generation of systemic protective immunity to LVS after sublethal infection is indeed specific for LVS itself.

As already mentioned, the rapid generation of protective immunity to LVS after sublethal priming is not consistent with the usual time requirements for activation, clonal expansion, and cytokine production by conventional T cells. Instead, we suggest that skin-related infection (39) may lead to efficient and rapid activation of keratinocytes (1, 20), γ/δ$^+$ dendritic epithelial T cells (42), and/or NK cells (13, 23). Keratinocytes can produce TNF (20), while dendritic epithelial T cells (42), NK cells (13, 23), and human peripheral γ/δ T cells (11) have the capacity to produce IFN-γ when stimulated by bacteria. In fact, in vivo depletion of γ/δ T cells exaggerates early *Listeria* infection (15), and in vivo

depletion of NK cells interrupts generation of immunity to *S. typhimurium* (38). While there is no evidence to date suggesting an antigen-specific activity for NK cells, the specificity and activation of requirements of γ/δ T cells are not yet understood. Early production of cytokines by such cells would most likely be indeed nonspecific, as suggested by in vitro studies demonstrating that stimulation of NK cells with many bacteria can lead to IFN production (13, 23), but would be sufficient to control initial bacterial replication. This short-term control mechanism would permit time for activation, expansion, and expression of specific humoral and/or T-cell-mediated responses that would ultimately be responsible for long-term control of infection, clearance of bacteria, and development of protective immunity. Without eventual participation of specific responses, the effects of early cytokine activity against heterologous infections would be limited to relatively minor influences on organ burdens (see references 27, 30, and 47), small increases in LD_{50} or survival (see reference 19), or transient protection reflected only in increases in the mean time to death (Fig. 4). Early nonspecific effects would be especially difficult to detect when relatively aggressive bacteria are used as the nonspecific challenge. Here, *S. typhimurium* and *E. coli* replicate in vivo much more rapidly than *F. tularensis*, and replication may quickly overwhelm any nonspecific protective effects. Such an early, nonspecific bridge period has recently been proposed for early infections of mice with *L. monocytogenes* as well (24). Thus, our future studies will concentrate on the cells activated by i.d. LVS infection and the activities that they express, with the expectation that these studies will elucidate the cascade of events necessary to permit the early resistance to infection and generation of specific immunity described herein.

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